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Extraction of pesticides from soil using direct-immersion SPME LC-Tips followed by GC–MS/MS: Evaluation and proof-of-concept

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ABSTRACT

A new method was evaluated and developed for the analysis of pesticides in sandy-loam soil by direct-immersion solid phase microextraction (DI-SPME) followed by gas chromatography tandem-mass spectrometry (GC–MS/MS) determination. Ten pesticides were selected based on a literature survey of the compounds reported to be present in EU soils. The extraction was performed using SPME LC-Tips, a new SPME configuration with the coated fibers attached to a disposable and easy-to-handle micropipette tip, which was immersed into a soil slurry made by the addition of an aqueous solution to the soil sample. Ten experimental parameters were evaluated with a Plackett-Burman design, after which the extraction time and percentage of organic solvent in the aqueous extraction were optimized separately. The two fiber chemistries available (PDMS/DVB and C_{18}) were evaluated in parallel for the entire work. In the final method, slurry samples were made by adding an aqueous solution (6 % methanol v/v) to 2 g of soil. The fiber was conditioned and then inserted, for extraction, into the samples, stirred by a magnetic bar. Afterwards, the analytes were desorbed onto 100 μL of methanol. After the addition of analyte protectants (ethylglycerol, gulonolactone, and sorbitol) the extract was injected into the GC–MS/MS system. Isotopically labelled penconazole was used as internal standard. A calibration was performed by extracting spiked soil with analyte concentrations of 0.1–50 µg/kg. Coefficients of determination of the linear calibration were between 0.94–0.98 for the PDMS/DVB and 0.92–0.99 for the C_{18}. Limits of detection range between 0.01–10 µg/kg for the PDMS/DVB and 0.1–10 µg/kg for the C_{18}. Overall, the C_{18} analytically outperformed the PDMS/DVB but required a longer extraction time (120 min vs 75 min for the PDMS/DVB). This method allows automation and generates low residual toxic waste, having the potential to be introduced as a greener and simpler alternative to currently used sample preparation methodologies.

1. Introduction

Soil is crucial for sustaining life on land. Nevertheless, many soils around the world are contaminated with heavy metals and anthropogenic organic compounds [1,2]. The European Union (EU) is currently discussing a soil health law [3], in order to monitor soil quality and leverage its restoration if needed. Since EU soils have recently been found to contain very significant amounts of pesticide residues [2], a simple and effective method for their qualitative and quantitative analysis would be essential in drafting a monitoring and remediation plan.

The analysis of pesticides in soils is related to several of the sustainable development goals, namely: it promotes the safety and quality of agricultural production, food security and sustainable agriculture practices (Goal 2: Zero Hunger), it helps and supports the identification and mitigation of potential health risks associated with pesticide residues and thereby leverages human health and well-being (Goal 3: Good Health and Well-being), it contributes to prevent ecosystem pollution and the pollution of water bodies due to pesticide runoff and consequently supports the access to clean water (Goal 6: Clean Water and Sanitation) [4].

Several sample preparation methodologies have been developed for multiclass pesticide residue analysis in soil, but recently QuEChERS has become the most used and widespread [5,6]. Its low instrumental

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requirement (e.g. versus pressurized liquid extraction), simplicity, robustness, adaptability to in-house tailored methods and applicability to a wide range of analytes have made it a widely used extraction method, and it has been reported to outperform other techniques in metrological parameters and simplicity [6,7].

Nevertheless, QuEChERS is not a perfect technique: it generates significant amounts of waste *per* sample (albeit of relatively low toxicity). This is especially true because the conical centrifuge tubes are used disposably since their reuse is tedious and analytically not advised (due to the possibility of cross- and carryover contamination). Furthermore, like other multiresidue methods, it cannot be used to extract permanently ionic pesticides such asglyphosate, glufosinate ordiquat.

Solid phase microextraction (SPME) is a comparatively mature technique introduced in 1990 [8], which has found a remarkable array of applications. Headspace solid-phase microextraction (HS-SPME) is a solventless technique and has become the standard for volatile organic compound analysis in many areas of application [9], due to its high enrichment factor and simple operational and instrumental requirements when compared to classical alternative techniques such as static or dynamic headspace. For the analysis of low volatility compounds, direct-immersion SPME (DI-SPME) presents certain conveniences over traditional solid-phase extraction (SPE) in water analysis, being simpler to operate and greener, as it generates almost no waste and is potentially reusable [10,11].

SPME has been used in soil for the analysis of several pesticides by headspace sampling of a soil slurry made by adding water and sodium chloride to the sample (to promote salting-out and volatilization of non-polar compounds) [12,13]. Direct immersion into the soil slurry would result in reduced fiber lifetime and much increased analysis costs. Recently, tailor made SPME sorbent materials have been developed for the extraction of a wider range of pesticides [5]. Nevertheless, all HS-SPME methodologies require sufficient analyte volatilization into the headspace. Since most currently used pesticides are not sufficiently volatile, samples need to be heated, which reduces fiber adsorption and degrades thermo-labile analytes [5]. Several workarounds have been developed over the years, such as fiber cooling devices [14], but these techniques are operationally and technically much more complex than e.g. QuEChERS, which has prevented them from being used in routine analysis.

SPME extractions (direct immersion and headspace) often follow a complex equilibrium of the analytes between the fiber phase and sample matrix. Thus, due to being a non-exhaustive technique, quantitative methods employing SPME have traditionally been challenging. Matrix effects (changes in pH, competitive adsorption, *etc.*) influence the partition coefficients of the analytes and consequently influence the efficiency of their extraction [15]. The use of matrix-matched calibration as well as isotopically labelled surrogate standards (for mass spectrometric detection) is advised since standard addition is often too laborious and expensive [15].

Recently, a new configuration of SPME has become commercially available - the LC Tips (originally from Merck/Sigma-Aldrich and recently from Bruker under the commercial name “SPE-it tips”). In this configuration, the SPME fiber is attached to the end of a micropipette tip, which allows it to be used (typically) with 96-well microplates. These fibers were originally developed for biological sample analysis, but they permit the isolation of analytes from any aqueous solution. Unlike traditional SPME, after extraction, the analytes are desorbed onto an organic solvent (retro-extraction with acetonitrile or methanol) before being injected [16]. These new fibers are around thirty times cheaper *per* unit than traditional SPME assemblies (2023 prices from Sigma-Aldrich), which allows them to be used semi-disposably.

This work presents a novel application of DI-SPME, using the LC-Tips configuration, which attempts to overcome the limitations of traditional SPME when applied to the context of pesticide residue analysis in soil. Furthermore, it intends to provide a more sustainable alternative to currently used methodologies, in line with green analytical chemistry principles. For the extraction, a soil slurry is made with an aqueous solvent, and the extraction is performed under stir-bar agitation of the slurry. A Plackett-Burman experimental design was conducted to screen different variables in the extraction. Afterwards, the composition of the organic solvent and extraction time were optimized separately, and the final methodology was evaluated in terms of linearity, detection limits and repeatability.

In order to select the analytes for this study, we conducted a literature review of recent articles reporting on pesticide soil contamination within the EU and constructed a list of high priority analytes based on those most commonly found in EU soils, and those most used in Portugal from 2018 to 2022, determined by a monitoring campaign of used pesticide packaging [17]. The ten compounds selected for this study are presented in Table 1. Glyphosate and its metabolite aminomethyl-phosphonic acid (AMPA) were also singled out as especially important contaminants. However, due to their ionic nature (akin to amino acids), they are not expected to adsorb significantly to non-polar SPME fiber coatings and therefore were not considered for this work.

2. Experimental

2.1. Materials and chemicals

The ten pesticides (boscalid, diflufenican, epoxiconazole, indoxacarb, metalaxyl, metolachlor, metribuzin, penconazole, tebuconazole and terbuthylazine) were of analytical grade, obtained from Sigma-Aldrich (Steinheim, Germany). Penconazole-d7 was obtained from Toronto Research Chemicals (Toronto, Canada). The water used in the extraction was ultrapure, produced in a Milli-Q plus system from Millipore (Bedford, MA, USA). Methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade, obtained from Honeywell (Charlotte, NC, USA). Dichloromethane was GC–MS grade, purchased from Carlo-Erba (Emmendingen, Germany). The remaining materials were all purchased from Sigma-Aldrich, namely: C_{18} and PDMS/DVB SPME LC-Tips, 3-Ethoxy-1,2-propanediol, gulonolactone and D-Sorbitol (analyte protectants) of purity 98, 95 % and 99 %, correspondingly, anhydrous sodium acetate and acetic acid of >99 % purity.

Stock solutions for the ten pesticides were prepared in acetonitrile at 250 μg/mL, and kept refrigerated at −20 ºC, for at most 1 month. Dilutions for injection and soil spiking were performed in methanol. A stock solution of the three analyte protectants was prepared in methanol at 2000 μg/mL each. Penconazole-d7 was dissolved in dichloromethane at 200 ng/mL.

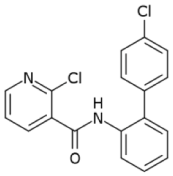
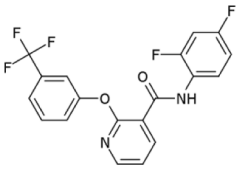
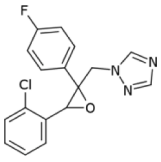
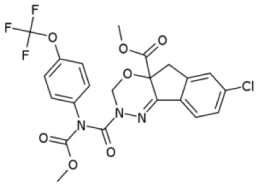
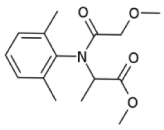
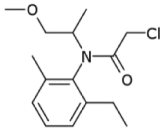
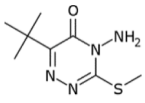
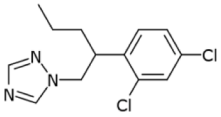
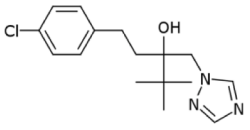
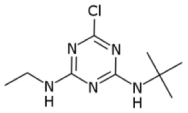
2.2. Soil sampling and spiking

The soil for the study was sampled from Idanha-a-Nova Municipality, in Eastern Portugal, from an area that was used in the past for tobacco culture (39,8454º N, 7,2544º W). Samples were collected at a depth of up to 25–30 cm. Once in the laboratory, they were sieved through a 2 mm mesh and allowed to air-dry at room temperature (~22º C), before being frozen at −20º C. Soil pH and pH_{KCl} ($n = 3$) were determined by mixing 4 g with 10 mL milli-Q water or 1 M of KCl, respectively, and shaken for 1 hour, prior to measurement.

The soil presents a sandy-loam texture (supplementary material, Table S1), 3.3 ± 0.3% organic matter ($n = 5$), pH of 7.73 ± 0.06, and pH_{KCl} of 6.94 ± 0.05. Soil metal content was also determined and can be found in supplementary material, Table S2.

For method development, the soil was spiked with the ten pesticides (Table 1) at 50 μg/kg. This was performed by adding an appropriate amount of solution to the soil (enough to disperse by capillarity, but not saturate), drying it under a nitrogen current to remove most of the solvent, and then allowing it to age for three days under aerobic conditions, after which it was kept at −20º C. A single spiked batch was used for all method development studies.

Table 1
Selection of compounds for this study, along with chemical structures and references which report their detection in EU soils. The references shown are those which report the compounds presence in soil. Logarithm of water-octanol partition coefficient (log K_{ow}) data from the Pesticide Properties Database [18].

Compound	Structure	Log K_{ow}	Action Type	Approval in EU**	CAS	References
boscalid		2.96	Fungicide	Aproved	188,425–85–6	[2,7]
diflufenican		4.2	Herbicide	Aproved	83,164–33–4	[7,17,19]
epoxiconazole		3.3	Fungicide	Not Approved	135,319–73–2	[2,7,19]
indoxacarb		4.65	Insecticide	Not Approved	173,584–44–6	[17]
metalaxyl		1.75	Fungicide	Aproved	57,837–19–1	[20]
metolachlor		3.4	Herbicide	Racemic mixture not approved, S-metolachlor (around 60 % enantiomeric excess) approved	51,218–45–2	[17,21]
metribuzin		1.7	Herbicide	Aproved	21,087–64–9	[17]
penconazol		3.72	Fungicide	Aproved	66,246–88–6	[17,22,23]
tebuconazole		3.7	Fungicide	Aproved	107,534–96–3	[7,17,19,20,23,24]
terbutylazine		3.4	Herbicide	Aproved	5915–41–3	[7,17,19–21,24]

** Data from the European Commission Website.

2.3. GC–MS/MS analysis

The analyses were performed by Gas Chromatography-Tandem Mass Spectrometry (GC–MS/MS) on a Bruker GC 456 and a Bruker Scion TQ (Triple Quadrupole) system equipped with a CTC CombiPAL automatic injector and a programmable temperature vaporizer (PTV) inlet (Bruker 1079). Data were acquired with Bruker MSWS 8.2 and analysed with Bruker MS Data Review 8.0. Chromatographic separation was achieved on a ZB-5MS Plus capillary column (20 $m \times 0.18$ mm i.d., 0.18 μm df). The oven temperature program began at 50 ºC held for 3 min, raised at 20 ºC/min to 140 ºC, then 4 ºC/min to 250 ºC, and finally 20 ºC/min to 310 ºC held for 2 min. Helium of 99.9999 % purity was used as carrier gas at a constant flow rate of 0.7 mL/min. The injection volume was 5 μL, performed in PTV large volume mode, starting at 80 ºC with a split ratio of 1:120, held for 30 s, then splitless and a temperature increase of 200 ºC/min to 270 ºC. At 3 min, the split valve was opened at a ratio of 1:60, and after 3 more minutes reduced to 15 mL/min and held for the entire run. The mass spectrometer system was operated in multiple-reaction monitoring (MRM), with argon as collision gas at 2.4 mTorr. The transfer line was held at 300º C, and the ion source at 270º C. The solvent delay was set to 15 min. MRM transitions associated with the selected precursor and product ion pairs of the analytes, their relative ratios, and a GC–MS/MS chromatogram of the standards can be found in supplementary material, Table S3 and Figure S1, respectively. The quadrupoles were operated at unit resolution, and the ion ratios between quantifier and qualifier had to be within ±30 % the average of injected standards for positive identification [25].

2.4. SPME-Tips extraction method development

For the extraction, 2 g of soil sample were weighted into 16 mL with 22 mm diameter vials (Supelco, Steinheim, Germany), and then the aqueous solution (water with an organic modifier, MeOH or MeCN, in different concentrations) was added to create the soil slurry (Mili-Q water with 1–10 % (v/v) of MeOH or MeCN). The vial caps had pierced septa where 200 µL plastic micropipette tips were placed as holders for the SPME LC-Tip fiber (Fig. 1). Before each extraction, the fibers were conditioned by inserting them in a 2 mL glass vial (Chromacol, Thermo Fisher Scientific, MA, USA) with 100 % organic solvent (either MeOH or MeCN) for 30 min, followed by re-equilibration in another 2 mL glass vial with the aqueous solution (water with an organic modifier, MeOH or MeCN, in different concentrations) for another 10 min, always under agitation on a Bunsen AO 400 mechanical linear/orbital shaker at 250 RPM's. Afterwards, the SPME LC-Tip fibers were immediately inserted into the soil slurry (without drying), which was stirred using a magnetic bar. After extraction, the fibers were removed and retro-extracted to 100 μL of the same organic modifier as the one used in the aqueous solution (MeOH or MeCN), using 300 μL insert glass vials (Alwsci Technologies, Zhejiang PR, China), under agitation at 250 RPMs. Every step of the extraction was performed at room temperature (22 ºC).

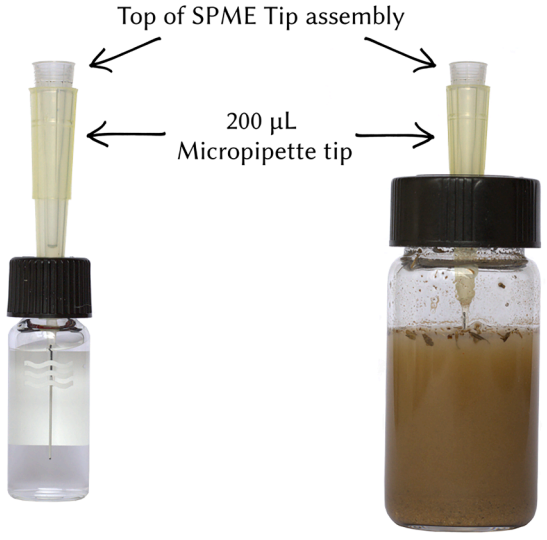


Fig. 1. Experimental setup for soil extraction using SPME-LC tips: (a) conditioning step, using 2 mL vials, (b) extraction step, employing magnetic stir-bar agitation. Retro-extraction is performed similarly to conditioning, except it uses vials with 300 μL inserts.

After initial experimentation, ten variables were determined for screening, to gauge their effect on method efficiency. A twelve experiment Plackett-Burman design was used for this purpose. The factors (variables) were: extraction time (30 or 60 min), use of a buffer (0.02 g of sodium acetate *per* mL of solvent and extraction aqueous solution with 1 % v/v acetic acid, values adapted from Lehotay et al. [26]), fiber coating chemistry (C_{18} versus PDMS/DVB), sonication of the slurry (prior to extraction, for 5 min, using Bandelin Sonorex Super RK 102 H, Berlin, Germany), salting-out (NaCl at 0.03 g/mL of aqueous solution, added to the dry soil), percentage of organic modifier (1 or 10 %), type of organic modifier (MeOH or MeCN), volume of solvent (10 or 12 mL, always for 2 g of soil), retro-extraction time (10 or 30 min) and concentration of analyte protectants [27] (250 or 500 µg/mL). Each experiment was performed in triplicate, and the SPME fibers were rotated randomly between experiments to prevent bias; twelve fibers were used, each three times. The main effect of each factor was calculated using the average of the three replicates of each experiment, and then tested for significance against a dummy factor (the eleventh variable in the design) using a one-tailed F-test at 0.05 significance level [28]. After the Plackett-Burman screening, the percentage of organic modifier and extraction time were further optimized for both fiber chemistries using the same spiked soil, whilst all other variables were fixed (Section 3.2).

2.5. Final extraction method

The final method was tested for both fiber chemistries in parallel. Samples were prepared by weighing 2 g of soil onto the extraction vial and adding 200 µL of internal standard solution (200 ng/mL in dichloromethane), which was allowed to air-dry for 30 min. Fibers were conditioned by being inserted in a 2 mL glass vial containing methanol for 30 min, followed by re-equilibration in another 2 mL glass vial containing water with 6 % methanol (v/v) for 10 min, under constant agitation at 250 RPMs. Afterwards, they were immediately exposed to the soil slurry, which was prepared by adding 10 mL of water with 6 % methanol (v/v) to the soil sample. The only operational difference between the fiber chemistries was the extraction time: 75 min for PDMS/DVB and 120 min for C_{18} (supplementary material, figures S2 and S3). After the extraction, the fibers were immediately inserted into a conical vial with 100 µL of methanol for 30 min at constant agitation (250 RPMs). From this methanolic extract, 60 µL were removed from the top (to eliminate any sediment deposited at the bottom, transported with the fiber) to another conical vial, and 20 µL of analyte protectant solution was added (to a final concentration of 500 µg/mL each), before analysis by GC–MS/MS.

2.6. Method validation

The calibration was performed for both C_{18} and PDMS/DVB fibers, by spiking the soil within the extraction vial with the ten pesticides at concentrations of 0.1–50 µg/kg (seven concentrations), and drying under a nitrogen stream, before following the final extraction method. For every concentration level, two different fibers were used, each two times, for a total of four extractions *per* concentration. Limits of detection (LoD) and quantification (LoQ) were determined visually for the qualifier MRM transition of each analyte (which had a worst signal-to-noise ratio than the respective quantifier) in the following way: the

lowest calibrated concentration with a signal-to-noise ratio above 3 (and clear peak identification) was assumed as the LoD, and above 10 was assumed as the LoQ. These limits were also calculated with a different method, from the calibration curve [29] (using the quantifier signals with higher signal-to-noise ratios), and the results can be found in the supplementary info, Table S4. Mandel's test was performed to judge the linearity of the calibration data [30]. Coefficients of determination for linear and quadratic regressions can be found in supplementary info, Table S8.

For estimating the mass of each analyte extracted, a calibration curve was performed by preparing six solutions in methanol with concentrations ranging from 0.1 to 40 ng/mL, and the same amount of analyte protectants as the extracts (500 μg/mL). These solutions were injected with the same method as the extracts. Least-squares fitting was performed in absolute units (no internal standard). The analyte mass injected into the GC–MS/MS was then calculated for the extracts of spiked soil at 10–30 μg/kg and transformed into a percentage of the total analyte mass in that spiked soil sample.

3. Results

3.1. Initial screening of variables

The first step was to evaluate the methodological operability of the extraction. SPME LC-tips were originally developed for biological samples on well trays, which allow process automation (*e.g.* robotic liquid handling systems), but this would not work for soil due to the larger sample amount and greater agitation required. The preliminary trials (data not shown) allowed us to determine that to produce a homogenous slurry (rather than soil deposition at the bottom), magnetic stir-bars were ideal, as opposed to shaking the entire vial. This also had the advantage of leaving the vial and fiber assembly static. Nevertheless, a very strong vortex is not advised, as it can leave the fiber exposed to air rather than the slurry, or damage it. The only disadvantages of using the stir-bars were (1) a more labor-intensive method and (2) increased strain on the materials, caused by the abrasive nature of soil particles, resulting in visible wear on both the vials and stir-bars. Still, observation of the fibers, which were located above the stirring bars, showed that they had good elasticity, and no visible mechanical damage was observed.

The first screening of variables was performed with a Plackett-Burman design (Section 2.4). These variables were chosen based on what were thought to be the influencing factors in the extraction. The results are presented in Table 2. The main effects were also calculated using the relative standard deviation of each experiment ($n = 3$), and tested for significance; However, only three significant values were present, at 0.05 significance level: diflufenican had a lower relative standard deviation (RSD) with no addition of salt, and both diflufenican and boscalid had lower RSD at 1 % organic modifier. Non-significant values can be found in the supplementary material, table S5.

The experimental results for some of the variables were similar across all analytes, namely acetate buffer, salting out and sonication, resulting in the exclusion of these steps from the extraction process. It was thought that increasing the ionic force of the slurry (salting-out) might potentiate the migration of analytes to the fiber. However, the results show the opposite, probably because the extraction from the soil to the aqueous solution was hampered. The buffer also increases the ionic force, and the neutral acetic acid molecules might compete for the fiber, especially the PDMS/DVB coating, further reducing extraction ability. It must be noted that, especially with alkaline soils, the use of a buffer may be preferable since several pesticides are known to degrade at high pH levels [26]. Furthermore, an unbuffered method will not be as robust to changes in soil chemistry.

A concentration of 500 μg/mL in the injected extract was used because it performed better overall (Supplementary material, table S5), even though most of these values were not statistically significant at 0.05 significance level. Nevertheless, a higher analyte protectant concentration should be beneficial for more polar and difficult analytes.

Fiber chemistry extraction performance varied among analytes, which is expected as they exhibit different polarities. Proper choice of fiber is one of the more important variables to consider for a specific pesticide class. Since this method aimed at analyzing several pesticide classes, further optimization was performed with both fiber chemistries.

According to the experimental data, at 30 min of extraction, no analyte had achieved the partition equilibrium point between the sample matrix and the extraction phase. Thus, the extraction time was one of the variables that needed further optimization. In terms of retro-extraction time, most analytes showed better signal and lower RSD at 30 min, but without statistical significance. A higher stirring during retro-extraction would likely improve desorption kinetics, but this was not necessary and could also create a vortex, causing the top portion of the fiber to not fully submerge into the solvent (as referred before).

The solvent volume was not a relevant variable for most analytes. Although a higher solvent volume increases the pesticide extraction from soil, it shifts the fiber adsorption-desorption equilibrium in the opposite way. A 10 mL volume was chosen for further studies because it was operationally simpler and produced slightly less waste.

3.2. Organic modifier and extraction time

The results of the Plackett-Burman screening point to a correlation between the modifier and its percentage in solution, with better results being achieved for 1 % methanol and 10 % acetonitrile. Methanol was selected primarily because the analyte protectants added before GC analysis have a low solubility in acetonitrile (especially the two sugars), but also because it showed better repeatability (Supplementary material, table S5).

The organic modifier may create different mechanisms within the extraction process: it favors extraction of the analytes from the soil

Table 2
Results of the Plackett-Burman screening. The values displayed are those which yield the highest signal for the average of three replicates. Non-significant main effects at 0.05 significance level are displayed by "-". Analytes are shown by elution order on GC–MS/MS (terbuthylazine first, indoxacarb last).

Analyte	Extrac. time (min)	Buffer	Fiber	Sonication	NaCl	%Organic modifier	Organic modifier	Solvent Volume (mL)	Retro-extrac. Time (min)	An. Protectant (mg/L)
terbutylazine	60	No	PDMS/DVB	–	–	1 %	MeOH	–	–	–
metribuzin	60	–	PDMS/DVB	No	No	1 %	MeOH	10	–	250
metalaxyl	–	–	–	–	–	1 %	–	–	–	–
metolachlor	60	–	–	–	–	–	–	–	–	–
penaconozole	60	No	–	–	No	10 %	–	–	–	250
tebuconazole	60	No	C18	–	No	–	–	–	–	–
diflufenican	60	–	–	–	–	–	–	–	–	–
epoxiconazole	60	No	C18	No	No	10 %	MeCN	–	–	500
boscalid	60	No	–	–	No	–	–	–	–	–
indoxacarb	60	–	–	–	–	10 %	MeCN	–	–	–

(increasing total extraction and equilibrium speed), it reduces the adsorption towards the fiber by increasing analytes solubility in the liquid phase, and it may compete with the analytes for the fiber. It remains unclear whether the organic modifier had a greater effect on the kinetics (speed) or thermodynamic equilibrium (overall "extraction").

The percentage of organic modifier (methanol) was tested with a 60 min extraction time and 30 min retro-extraction. The results are presented in Fig. 2 for both C_{18} and PDMS/DVB fibers.

The most polar compounds (metalaxyl and metribuzin) showed a significant decline in extraction efficiency for both fibers as the organic modifier concentration increased. This may be due to a reduced extraction from the soil or more likely because of reduced fiber absorption/adsorption. However, considering that most analytes have a similar behavior with both fiber coatings (C_{18} and PDMS/DVB), it can be inferred that the methanol concentration mostly modifies soil extraction step rather than the fiber adsorption-desorption equilibrium (although it is likely to have an effect here as well). This is also supported by the fact that, in general, less polar analytes' extractions were favored by higher methanol volumes. If fiber competition between the analytes and methanol was the most significant factor, this latter effect was not likely to be observed. Overall, the results show that no value will accommodate all chosen analytes due to their different chemical properties (*e.g.* polarity). A methanol concentration of 6 % (v/v) was chosen for both fibers.

The extraction time was evaluated for both fibers (supplementary material, figures S2 and S3). For the PDMS/DVB, most analytes reached the extraction maximum point at 75 min, followed by a small decrease. Thus, this extraction time was chosen. Concerning the C_{18} fiber, in the time range evaluated (30–120 min), no analyte was observed reaching the equilibrium point, except for diflufenican (which reached maximum extraction at 90 min). Thus, 120 min was selected in order not to extend the extraction time beyond two hours. This was done firstly to avoid an excessively long extraction (as the abrasion causes damage to the vials and stir-bar) and because some degradation-prone analytes should not be in aqueous solution for too long, especially considering the increase in temperature caused by the stir-bar, which was uncontrolled although experiments were performed at 22º C. At 120 min, the temperature was around 30 - 32 ºC, $n = 20$.

3.3. Carryover and clean-up

No instrumental carryover was detected in any blank run (MeOH injection) across the entire concentration range of the analyzed samples, even after the injection of the highest extracted concentrations. Fiber carryover was investigated firstly by retro-extracting the same fiber a second time (to a fresh 100 μL of MeOH) after the extraction of a soil with 50 µg/kg of each analyte. The second extract still had detectable amounts of nearly every analyte in both fiber chemistries, albeit low (under 10 % signal intensity compared to the 50 µg/kg extract). However, it was found that conditioning the fibers after extraction from soil (30 min in MeOH and 10 min in water with 6 % methanol) was sufficient to remove any detectable carryover. Consequently, in terms of carryover no opposition was found to re-using the SPME fibers, but since the conditioning solvent itself should not be reused (as it may concentrate carryover), it adds significantly to the overall solvent waste per sample.

A simple clean-up of the SPME fibers was tested: after extraction, the fibers were removed and inserted into 100 μL of Mili-Q water for 2 min, and agitated at 250 RPM, after which they were immediately retro-extracted accordingly to the methodology described above (Section 2.5). Although no analyte loss was observed (values within signal interval for that concentration), the method was insufficient to clean the fiber of attached matter (usually suspended organic matter from the soil which had clung to the fiber). Thus, after retro-extraction it was still necessary to let the extract settle and collect only 60 μL from the top, to ensure no solid matter was in the injected extract. Furthermore, the extract without clean-up was rather free of interferents, both in the MRM chromatogram and full-scan chromatogram when it was performed (supplementary material, Figure S4). Following this experimental observation, it was assumed that the fibers coatings would not be prone to carry any significant amount of matrix interferences (*e.g.* dissolved metals or soil colloids) and the clean-up step was considered unnecessary. The largest non-analyte peak observed in the MRM chromatogram was present in most samples and blanks (Fig. 3, number 10), possibly originating from the plastic micropipette tips used to measure volumes and hold the LC-tips. In terms of the analyte protectants, since the amount used in this work was quite large (mass equivalent to 2.5 mg/mL for a 1 µL injection), the use of GC-grade analyte protectant standards was desirable, but the authors were unable to find any commercially available.

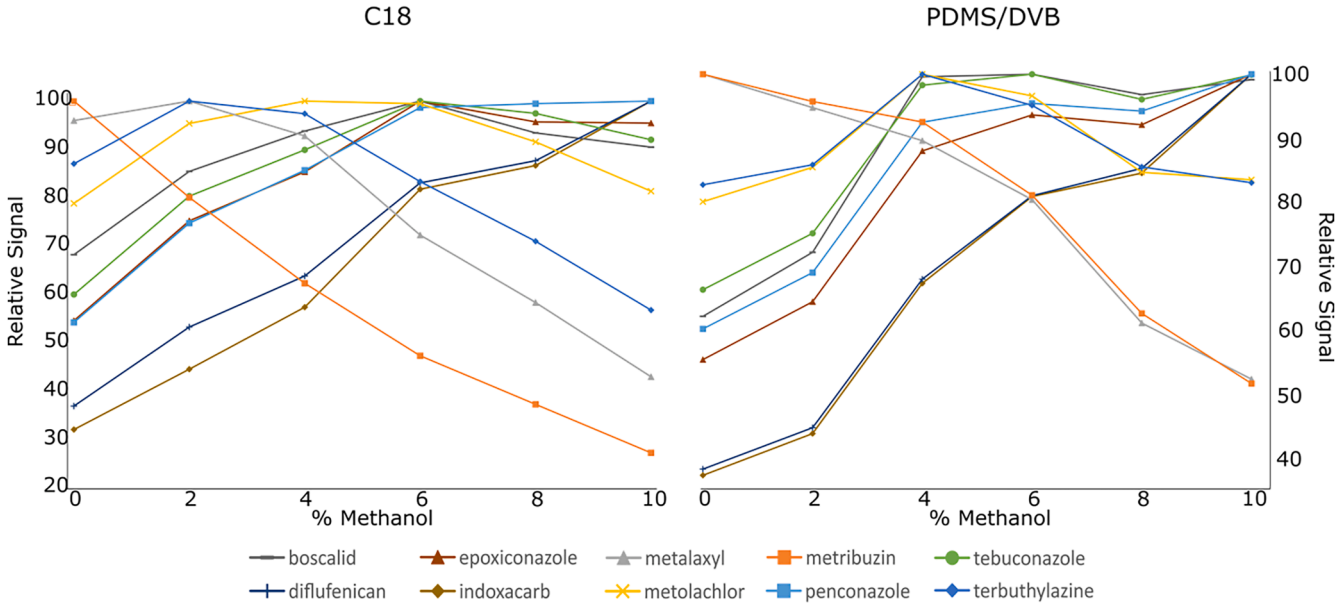


Fig. 2. Analytes extraction according to the percentage of methanol in the aqueous solvent. The highest absolute signal for each analyte was normalized to 100 %, and the remaining were scaled accordingly.

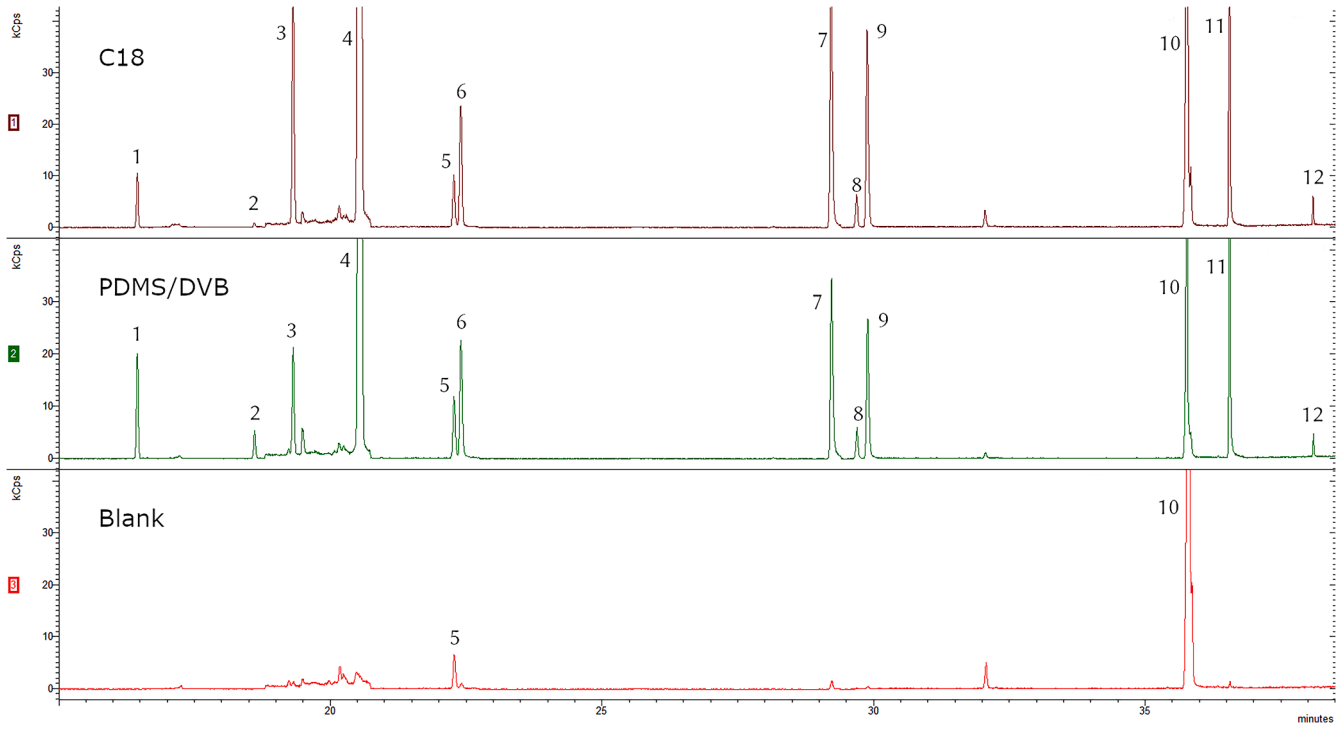


Fig. 3. Example MRM chromatograms (sum of quantifier and qualifier), displaying the analytes. Top and middle are soil extracts with 10 μg/kg spike for each analyte, using the final extraction method (Section 3.5). Bottom is an extracted blank using the PDMS/DVB fiber, from soil with internal standard only. 1 – terbuthylazine, 2 – metribuzin, 3 – metalaxyl, 4 – metolachlor, 5 – penconazole-d7 (Int. standard), 6 – penconazole, 7 – tebuconazole, 8 – diflufenican, 9 – epoxiconazole, 10 – unknown compound, 11 – boscalid, 12 – indoxacarb.

3.4. Calibration and method repeatability

The calibration results for both fibers are presented in Table 3. Every analyte in every sample had a quantifier/qualifier ion ratio within the acceptable range, except those that fell below the LoD. For the linearity study, Mandel's test was performed, which compares the residual's variance for the linear and quadratic fittings [30]. For the PDMS/DVB fiber, every calibration except that of metalaxyl was better modeled with a quadratic regression, whereas for the C_{18} fiber, this was only the case for tebuconazole and indoxacarb. Thus, it appears that C_{18} calibrations presented better linearity, whereas the PDMS/DVB, when calibrated at this concentration range, is better fitted by a quadratic equation. However, this phenomenon cannot be explained by fiber saturation, as the curves for every quadratic fitting tended upwards rather than towards a maximum signal. . For nearly every analyte, the C_{18} fiber showed better coefficients of determination for the linear fitting, and similar for the quadratic fitting (data in supplementary info Table S8).

In terms of detection and quantification limits, the C_{18} extraction performed equally or better except for metribuzin, penconazole and tebuconazole. These analytes (apart from penconazole), have significant polarity when compared to the remaining ones. Interestingly, even though terbuthylazine seemed to favor the PDMS/DVB fiber in terms of signal when tested during the Plackett-Burman screening (at 50 μg/kg), detection and quantification limits were lower with the C_{18} fiber. Overall, the results are congruent with the Plackett-Burman screening, in that the C_{18} fiber coating performed generally better. However, it must be noted that most analytes in this study are relatively non-polar within the spectrum of currently used pesticides, and that any further experimentation of more polar analytes that require LC-MS analysis, may achieve better results with the PDMS/DVB fiber. Also, when evaluating extraction time, a concentration of 50 μg/kg was used, and the fact that the longer extraction time used for the C_{18} fiber favors the extraction of lower analyte concentrations needs to be considered.

The addition of the analyte protectants before GC–MS/MS analysis resulted in a slight dilution of the extract, which directly influenced the limits of detection. However, for some analytes at low concentrations, the protecting effect greatly improved peak shape. Furthermore, since the extract was relatively clean, a larger-volume injection may have been considered, aiming to lower the LoD.

The repeatability of extraction replicates was quite poor. Relative standard deviations for six extractions were as high as 50 %, and generally in the 10–40 % range. The internal standard was very effective at mitigating the lack of precision, especially at concentration ranges close to its own (5–16 % RSD for 20 μg/kg). Regardless, calibration of a wider concentration range might result in significant heteroscedasticity; thus, the internal standard concentration must be adjusted to account for this factor. Because each calibration point has a significant number of preparation steps (weighed, spiked, dried, etc.), there is also room for random operator errors which might have compromised the values in

Table 3
Method performance parameters for the C18 and PDMS/DVB fibers using the final method as described in Section 2.5. R^2 values are of the linear regression. The asterisk denotes the calibrations in which the null hypothesis was rejected at 0.05 significance level for the Mandel test i.e. when the data is "better" fitted by a quadratic regression than a linear one.

Analyte	R^2		LoD		LoQ	
	PDMS/DVB	C18	PDMS/DVB	C18	PDMS/DVB	C18
terbutylazine	0.972*	0.988	10	1	20	10
metribuzin	0.965*	0.950	1	10	10	20
metalaxyl	0.952	0.970	1	0.1	10	1
metolachlor	0.976*	0.996	0.1	0.1	1	1
penconazole	0.975*	0.997	0.1	1	1	10
tebuconazole	0.963*	0.974*	0.01	0.1	0.1	1
diflufenican	0.948*	0.971	0.1	0.1	1	1
epoxiconazole	0.959*	0.986	1	0.1	10	1
boscalid	0.942*	0.987	1	0.1	10	1
indoxacarb	0.957*	0.915*	10	10	20	20

terms of repeatability, especially drying the spiked samples under nitrogen, where a sufficiently high flow could cause the finer soil particles to be blown off. Also, the SPME extraction itself is known to generate high replicate deviations, and thus its applicability in quantification has traditionally been difficult [15]. This phenomenon might be further aggravated by fiber variability, as this method uses different fibers (each used up to three times) rather than a single one for all extractions like traditional SPME assemblies. Consequently, it is unlikely that this type of DI-SPME will provide adequate repeatability without internal standard correction. Lack of repeatability could also be partly explained by the non-controlled temperature increase inside the extraction vial, as detailed above. The extraction temperature was not controlled because that would make the system more complicated, and thus less able to compete with relatively straightforward extraction methodologies such as QuEChERS. However, in the future this might be an interesting avenue for further optimization. Increasing the extraction temperature will reduce fiber adsorption but might increase the extraction from the soil onto the solvent, although this would not be a viable option for degradation-prone analytes. Finally, changing the sample size is likely to have an effect on both repeatability and detection limits. Although we found little statistically significant difference between an extraction of 10 or 12 mL from a 2 g soil sample, either miniaturizing the extraction (using for example 0.5 g of soil and 2.5–5 mL of solvent, possible due to the fiber's small size), or maintaining the solvent volume whilst increasing sample mass, could change these parameters. Unfortunately, miniaturization is known to have a negative effect on repeatability and sample representativeness, but for this method in particular it could result in a more homogenous extraction, if adequate stirring is provided.

The same fibers were used up to three times. The variability between replicates of different fibers (in absolute signal) was generally higher than the variability between subsequent extractions of the same fiber. Consequently, any elations as to fiber degradation/loss of efficiency were difficult, but no consistent proof of lower extraction efficiencies for second and third extractions was found. In terms of the calibration curve, due to internal standard correction, there is no significant difference in values from the first to third extractions of the same fiber. Furthermore, since they are relatively cheap, from a monetary perspective there is no need to reuse the fibers more than three times.

To roughly estimate the percentage of analyte being extracted onto the fibers, a calibration curve was constructed by injection of the analyte standards (described in Section 2.6). Every analyte had an R^2 above 0.99, and every instrumental LoQ was below 0.1 ng/mL except for indoxacarb, which was 1 ng/mL. The absolute signals from the soil extractions were translated into% of analyte extracted from the soil, considering the full mass of analyte present in the 2 gs of soil (full data in supplementary material, Tables S6 and S7). The C_{18} fiber performed better for every analyte except terbuthylazine and metribuzin, with an average extraction of 2.46 % (0.29 - 5.62 %) for 10–30 μg/kg concentrations, whereas the PDMS/DVB fiber had an average of 1.29 % (0.20 - 3.12 %). Since SPME is a non-exhaustive technique, these results show that, as expected, most of the analyte mass is not extracted onto the fiber. Furthermore, since this method utilizes a retro-extraction step prior to injection, it will never achieve as great a concentration factor as traditional SPME with direct fiber thermal desorption, since only a small percentage of the extract volume is injected.

3.5. Evaluation under green analytical chemistry

In order to evaluate the methodology, two different metrics were calculated: AGREE [31] and Analytical Eco-Scale [32]. The results were compared to twelve other methodologies for multiresidue analysis of pesticide residues in soil, whose values had been previously calculated and reported in a review [6]. These methods involve several types of extraction (pressurized liquid extraction, QuEChERS, solid-liquid extraction, microwave-assisted extraction) and gas or liquid chromatography coupled to various mass spectrometers (quadrupole, triple quadrupole, high-resolution tandem mass spectrometers). We have calculated these metrics exactly as described in that review [6]. Since the methods for C_{18} and PDMS/DVB differ only in the extraction time, which is not the bottleneck in terms of overall time (as several samples can be extracted concurrently), both methods yielded the same values.

For AGREE, a value of 0.46 was obtained (detailed in supplementary material, Figure S5). For the Analytical Eco-Scale, the result was 81. These values are tied to the highest ones obtained for the twelve methodologies previously evaluated (0.29–0.46 for AGREE and 58–81 for analytical eco-scale) [6]. Clearly, the method scored high in terms of green analytical chemistry principles but did not greatly outperform others. The greatest contribution for this good performance is the fact that water is used as the extraction solvent, although the small amount of methanol cannot eliminate dangers such as flammability and human toxicity, which is significant for this solvent. Another advantage over currently used methodologies is that it is operationally simple, involving a reduced number of steps and no drying under nitrogen or other complex procedures. Nevertheless, the method determines a relatively small number of analytes compared to other multiresidue methodologies, which reduces its performance. The "green" character of the fibers themselves is very difficult to judge, as there is no information about its manufacture, i.e. how much energy it uses, the generation of toxic effluents, etc. Using the fiber's low cost as a proxy might imply that the energy and waste cost is not too great, but this could obviously be misleading. In this respect, reusing the fibers is the best way to reduce ill effects from their manufacture, and as stated above, we have found them to be usable at least three times without any significant loss in performance parameters.

In the future, the greatest improvement in terms of green analytical chemistry performance would be attainable by the replacement of methanol with ethanol, both in conditioning and extraction. Besides (mostly) eliminating human toxicity, ethanol can be easily obtained from bio-based feedstocks. However, it is still flammable, and being a controlled substance might be difficult to obtain in some countries. For use in retro-extraction and injection, ethanol's high boiling point might pose a problem for traditional splitless injection, and the low solubility of the analyte protectants might also be a problem, which would be mitigated by co-dissolution with methanol. Nevertheless, ethanol could improve the retro-extraction of non-polar analytes over methanol and maybe allow the analysis of pesticides such as organochlorines with this technique.

4. Conclusion

In this study, a new methodology for the extraction of ten pesticides from soil was evaluated. The final method proved successful in terms of the preliminary validation performed and yielded acceptable detection limits. The main advantages of this methodology are the possibility of automation (to a large extent), low capital costs *per* sample and especially low generation of toxic wastes, thus providing a more sustainable alternative to currently used methodologies. Notwithstanding, it still suffered from relatively poor sample repeatability, which was compensated by the isotopically labelled internal standard. Of the two fiber chemistries commercially available, C_{18} performed best in most parameters, except for the more polar analytes (metalaxyl and metribuzin).

Given the inherent variability in the world's soils, an extrapolation of this method's effectiveness to other soil types (notably with finer textures, such as clay) cannot be inferred. Thus, in the future, it would be interesting to study and evaluate the applicability of this method for various soils, as well as expand it for the analysis of other pesticides. It is common knowledge, however, that no specific method will be able to cover all analytes under ideal conditions, but in-house and customized/tailored methods can be designed and evaluated. Therefore, if a more finely tuned method is required (*e.g.* to achieve lower LoDs), these may be validated for a specific class of pesticides in certain matrices and

conditions. According to the experimental data, the most important variables to evaluate are fiber chemistry, extraction time and composition of the extraction solvent.

CRediT authorship contribution statement

João Brinco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Raquel Carvalho:** Methodology, Investigation, Formal analysis. **Marco Gomes da Silva:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Paula Guedes:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Alexandra B . Ribeiro:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Eduardo P . Mateus:** Writing – review & editing, Validation, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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